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Cylindrochytridium johnstonii is a member of the *Cladochytriales*

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Abstract — The taxonomy of the *Chytridiomycota* has been in flux between a classical system based on thallus morphology and a new system based on zoosporic ultrastructure and analyses of genetic sequences. *Chytridiales* sensu Sparrow has been divided into 7 orders plus undescribed lineages. We found and brought into pure culture *Cylindrochytridium johnstonii*, the type species of the genus, which heretofore has not been characterized by molecular methods. We confirmed that this species is a member of the *Cladochytriales*, but it does not lie within a recognized family.

Key words — nucLSU rDNA, nucSSU rDNA

Introduction

Molecular studies have led to new orders being segregated from the large chytridiomycete order *Chytridiales* sensu Sparrow (Barr 1980; Letcher et al. 2006, 2008; Longcore & Simmons 2012; Mozley-Standridge et al. 2009; Simmons et al. 2009), yet many genera and species were not represented in these studies because they were unavailable in culture. Such species, which were classified in the *Chytridiales* in the classical literature (e.g., Sparrow 1960, Karling 1977), remain incertae sedis in the *Chytridiomycetes*. Therefore, when chytrids that have not been studied with molecular tools are isolated into pure culture it is appropriate to determine their taxonomic position in the new system and provide photographs of developmental morphology. Reporting genetic data for described species is intended to promote universal taxon concepts. The phylum *Chytridiomycota* has previously relied on thallus morphologies and zoosporic ultrastructure, both of which, at least to some extent, have led to paraphyletic groupings (e.g. *Chytridiales* sensu Sparrow 1960, *Chytridiales* sensu Barr 1980) compared to molecular phylogenies (James et al. 2000, 2006).

The genus *Cylindrochytridium* was described by Karling (1941) for *C. johnstonii*, a species with unique developmental morphology that occurred on boiled grass used as bait in samples of aquatic debris. Subsequently, *C. johnstonii* has been

reported from sites around the world, including New Zealand (Karling 1968), South America (Marano et al. 2008a,b; Rocha & Pires-Zottarelli 2002), Europe (Czeczuga et al. 2007), and India (Karling 1963), but no authors have reported isolating this fungus into pure culture. Its phylogenetic position therefore has remained unconfirmed. We found this species growing on the edge of onionskin bait placed with algae and aquatic debris collected from Smith's Fen (Schwintzer 1978) during a field trip that was part of the 2008 centennial celebration of the University of Michigan Biological Station in northern Michigan, USA. Herein we report that *C. johnstonii* is a member of the *Cladochytriales* and support this conclusion with molecular data. Also, we include photos of the development of this chytrid in pure culture for comparison with the type description.

Materials & methods

Collection, isolation & morphology

JE Longcore collected algae and plant debris on 22 Sep 2008 from Smith's Fen (pH = 5.7; Schwintzer 1978), University of Michigan Biological Station, Cheboygan County, Michigan, USA. The sample was transported at ambient temperature in a Whirlpak® plastic bag to our laboratory in Maine. We placed the aquatic sample in a finger bowl at room temperature and baited it with pieces of boiled, white onionskin. After several weeks we noted thalli with catenulated rhizoids on the bait. Most of the thalli grew at the edges of the onionskin and produced long, cylindrical zoosporangia. We rinsed the onionskin with a stream of distilled water and placed it in a depression slide with distilled water. After ~1 hour, when many active zoospores could be seen, we added the water to plates of TC agar (0.4 g tryptone, 4 g cellobiose, 10 g agar, 1 L distilled water) containing 300 mg/L streptomycin and 200 mg/L penicillin G. We monitored the isolation plates at 100× magnification by inverting them on the stage of a compound microscope. After nearly two weeks, we selected *Cylindrochytridium johnstonii*, recognized by its long, cylindrical zoosporangia, from among the other chytrids on the isolation plate and removed thalli to clean plates of TC agar. We transferred the isolate, designated JEL596, to mPmTG slants (Longcore 1992) in screw-topped 125 × 20 mm culture tubes and maintained the stock tubes at room temperature. Stocks were transferred at 3-month intervals. To document morphology and days to maturity, we inoculated cultures on mPmTG agar in 9 cm Petri plates stored at 23 °C. To document morphology on cellulosic substrates, we inoculated a flask of sterilized lake water containing pieces of white onionskin with thalli near maturity or with motile zoospores present and incubated the flasks at room temperature. We aseptically removed portions of agar with thalli from plates and pieces of onionskin from flasks to view the chytrid by light microscopy. We photographed developmental stages with phase contrast and Hoffman Modulation Contrast (HMC) optics on a Nikon E400 microscope (Nikon Instruments, Melville, New York) equipped with a Spot RT digital camera (Diagnostic Instruments, Sterling Heights, Michigan). Composite images of thalli through multiple focus planes were assembled from overlapping micrographs of individuals in Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, California).

Table 1. Isolates of *Cladochytriales* used for phylogenetic analyses of *Cylindrochytridium johnstonii*

Isolate	Culture no.	Origin	Habitat	GenBank accession no.	
				nucSSU	nucLSU
Ingroup:					
Allochytridium expandens	Barr253	North Carolina, USA	Soil/detritus	AF164291S1	EU828501
Allochytridium luteum	Barr463	Ontario, CAN	Sandy soil/detritus	AY349047	AY349082
Catenochytridium sp. 1	JEL145	Maine, USA	Aquatic/*Eriocaulon*	EU828475	EU828503
Catenochytridium sp. 2	JEL024	Maine, USA	Aquatic/detritus	EU828476	EU828504
Catenochytridium sp. 3	JEL044	Maine, USA	Aquatic/detritus	EU828478	EU828506
Cladochytrium sp.	JEL153	Maine, USA	Aquatic/maple leaf	EU828458	EU828485
Cylindrochytridium johnstonii	JEL596	Michigan, USA	Aquatic/detritus	JF796051	JF796052
Diplophlyctis sp.	JEL331	Maine, USA	Aquatic/*Characeae*	EU828477	EU828505
Endochytrium ramosum	JEL402	Michigan, USA	Aquatic/*Cladophora*	EU828484	EU828513
Endochytrium sp. 1	JEL027	Maine, USA	Aquatic/*Eriocaulon*	EU828471	EU828498
Endochytrium sp. 2	JEL070	Maine, USA	Aquatic/*Eriocaulon*	EU828472	EU828499
Endochytrium sp. 3	JEL324	Maine, USA	Aquatic/*Elodea*	EU828473	EU828500
Nephrochytrium aurantium	JEL036	Maine, USA	Aquatic/*Sparganium*	EU828468	EU828495
Nephrochytrium sp. 1	JEL327	Maine, USA	Aquatic/*Nitella*	EU828467	EU828494
Nephrochytrium sp. 2	JEL125	Maine, USA	Aquatic/*Characeae*	AF164295S1	EU828511
Nowakowskiella elegans	JEL046	Maine, USA	Aquatic/detritus	EU828463	EU828490
Nowakowskiella elegans	JEL127	Maine, USA	Aquatic/*Characeae*	EU828466	EU828493
Septochytrium sp.	JEL177	Wales, UK	Aquatic/detritus	EU828474	EU828502
Septochytrium variabile	JEL191	Ontario, CAN	Aquatic/*Eriocaulon*	AH009045	EU828512
Unidentified sp.	JEL072	Maine, USA	Aquatic/*Eriocaulon*	EU828470	EU828497
Outgroup					
Karlingiomyces sp.	JEL093	Maine, USA	Aquatic	AF164278	AY349085
Polychytrium aggregatum	JEL109	Maine, USA	Aquatic	AY601711	AY349084

Sequencing & analyses

DNA of JEL596 was extracted with Whatman® FTA card technology (Whatman Ltd., Maidstone, Kent, UK; Borman et al. 2006, Simmons 2011). Segments of nucSSU and nucLSU rDNA were amplified, sequenced, and aligned as in Simmons (2011) with taxa from *Cladochytriales* (Table 1) after a BLAST search in GenBank grouped JEL596 within that order. A region of nucSSU rDNA was amplified with the NS1/NS4 primer pair (White et al. 1990), as was a region of nucLSU rDNA with the LR0R/LR5 primer pair (Rehner & Samuels 1994, Vilgalys & Hester 1990). The Akaike Information Criterion in jModeltest 0.1.1 (Guindon & Gascuel 2003, Posada 2008) chose the TIM3+G model of evolution for the combined dataset. We entered these parameters into MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003), which ran for 1M generations, selecting every 100th tree. A 50% majority rule phylogram was constructed and MP bootstrap and BPP values were computed in PAUP* 4.0b10 (Swofford 2002) as in Simmons (2011).

Taxonomy

Cylindrochytridium johnstonii Karling, Bull. Torrey Bot. Club 68: 383 (1941)

Figure 1

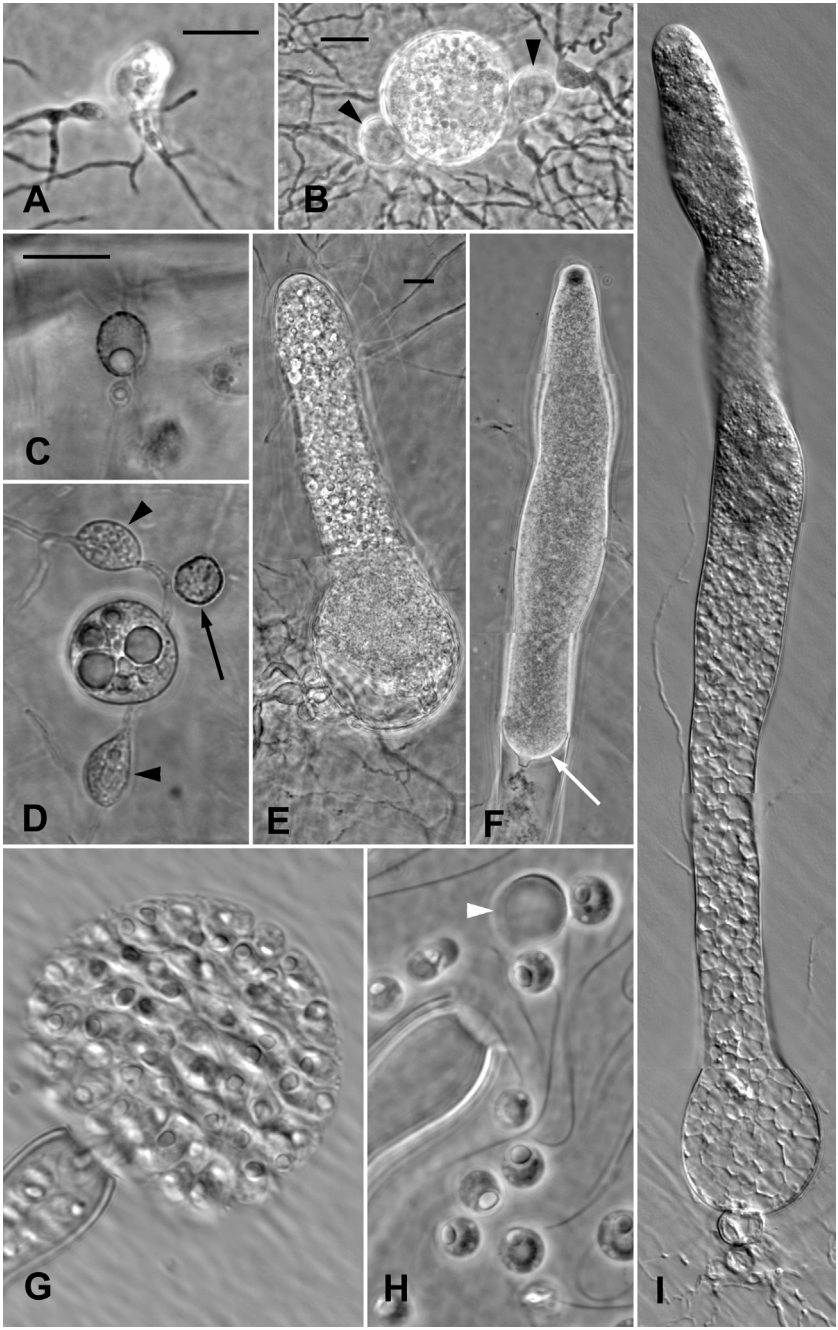
Type: Karling (1941: Figs. 1–16).

Specimen examined. **USA**. **Michigan**. On onionskin substrate added to water culture of algae and aquatic plant detritus, 22 Sep 2008. JEL596 (nucSSU rDNA sequence GenBank JF796051; nucLSU rDNA sequence GenBank JF796052).

We emend the description of *Cylindrochytridium johnstonii* by adding morphology on agar and axenic onionskin and molecular sequence information for JEL596.

Thallus characters — In pure culture, isolate JEL596 has morphological characters similar to Karling's (1941, 1977) descriptions of *C. johnstonii*. On agar, germlings developed endogenously, forming catenulate swellings at a single axis or multiple rhizoidal axes (Fig. 1A, B). The catenulations and the spherical base of what would become the zoosporangium developed relatively

Figure 1 (right). *Cylindrochytridium johnstonii* on mPmTG agar and liquid medium grown at 23 °C and on onion skin grown at room temperature. Micrographs taken with phase contrast optics unless otherwise noted. A. Germling at 3 days with catenulated rhizoids. B. Developing thallus at 8 days with two catenulated rhizoidal axes (arrowheads) and diffusely branching rhizoids. C. Brightfield micrograph of amber, ovoid zoospore cyst on onion skin with ridges along surface of cell wall and a single, large lipid globule. D. Brightfield composite micrograph of exogenously developing germling in onion skin, with zoospore cyst (arrow) and catenulated rhizoids (arrowheads). E. Composite micrograph of broad tube extending from spherical base of zoosporangium. F. Septum (white arrow) with tapered nipple delimiting zoosporangium base and apically migrating cytoplasm in thallus. G. Hoffman Modulation Contrast (HMC) micrograph of zoospores released from apical pore. H. HMC micrograph of spherical zoospores after release from zoosporangium by dehiscence of operculum (white arrowhead). I. Composite HMC micrograph of developing thallus with catenulated rhizoids, vacuolated portion of zoosporangium towards spherical base, and apically migrating cytoplasm. Scale bars = 10 μm; bar in A for G–H; bar in C for D; bar in E for F,I.


A
B
C
D
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G
H
I

slowly as the rhizoidal system expanded through the agar (Fig. 1B). After nearly two weeks, one side of the spherical zoosporangium elongated to form a tube nearly as wide as the spherical base (Fig. 1E). Most of the cytoplasm migrated toward the apical portion of the broad tube, leaving a highly vacuolated portion at the zoosporangium base (Fig. 1F, I). At maturity, the basal portion of the zoosporangium was empty and may have been walled off by a septum (Fig. 1F). Zoospores exited the zoosporangium through a pore made by the complete detachment of an apical operculum (Fig. 1H). Zoospores possessed a single lipid globule (Fig. 1G, H), and flagella were ~35 μm long.

Our attempts to reintroduce *C. johnstonii* to onionskin led to the production of germlings, but after 1 month, no further development occurred. On onionskin isolate JEL596 developed exogenously. The amber, ovoid zoospore cyst on the surface of the onionskin had a cell wall with ridges and generally possessed a single, large lipid globule (Fig. 1C). Germlings possessed one or two rhizoidal axes with catenulations (Fig. 1D).

Phylogenetics — A majority rule Bayesian phylogram (Fig. 2) was constructed from a data matrix with 337 parsimony-informative characters of a total matrix of 1410 characters. The phylogeny indicates that *Cylindrochytridium johnstonii* is within the *Cladochytriales*. The species is in a clade that is sister to a clade containing the type species of *Allochytridium* Salkin (Salkin 1970) and *Septochytrium* Berdan (Berdan 1939), which are the only two genera recognized in *Septochytriaceae* (Mozley-Standridge et al. 2009).

Discussion

Our phylogeny (Fig. 2) places *Cylindrochytridium johnstonii* as sister to an isolate with *Catenochytridium* morphology (JEL145). Together these two taxa are sister to a group of isolates in the *Septochytriaceae* (Mozley-Standridge et al. 2009) and are also part of a larger clade (Fig. 2), whose members form large catenulations in their rhizoids. One species in this clade, *Allochytridium expandens* Salkin, also has an elongated zoosporangium, but not to the extent seen in *C. johnstonii*. Additionally distinguishing these two taxa, *A. expandens* develops exogenously both in natural substrates and on agar medium (Barr 1986) whereas *C. johnstonii* develops endogenously on agar medium.

Shanor (1944) commented upon the resting spore of *C. johnstonii*, but he did not illustrate this feature. He described the resting spore on filter paper as being light-brown or amber, nearly spherical, with a smooth, thick cell wall and one large yellow oil globule. This description is very similar to that of the zoospore cyst that we saw on onionskin, but fine ridges appeared on the surface of most zoospore cysts we observed. Though the amber zoospore cysts on onionskin may look like resting spores, their connection to larger portions of the thallus (Fig. 1D) lead us to conclude they are zoospore cysts. Thus, we believe Shanor may have mistaken the zoospore cyst for a resting spore.

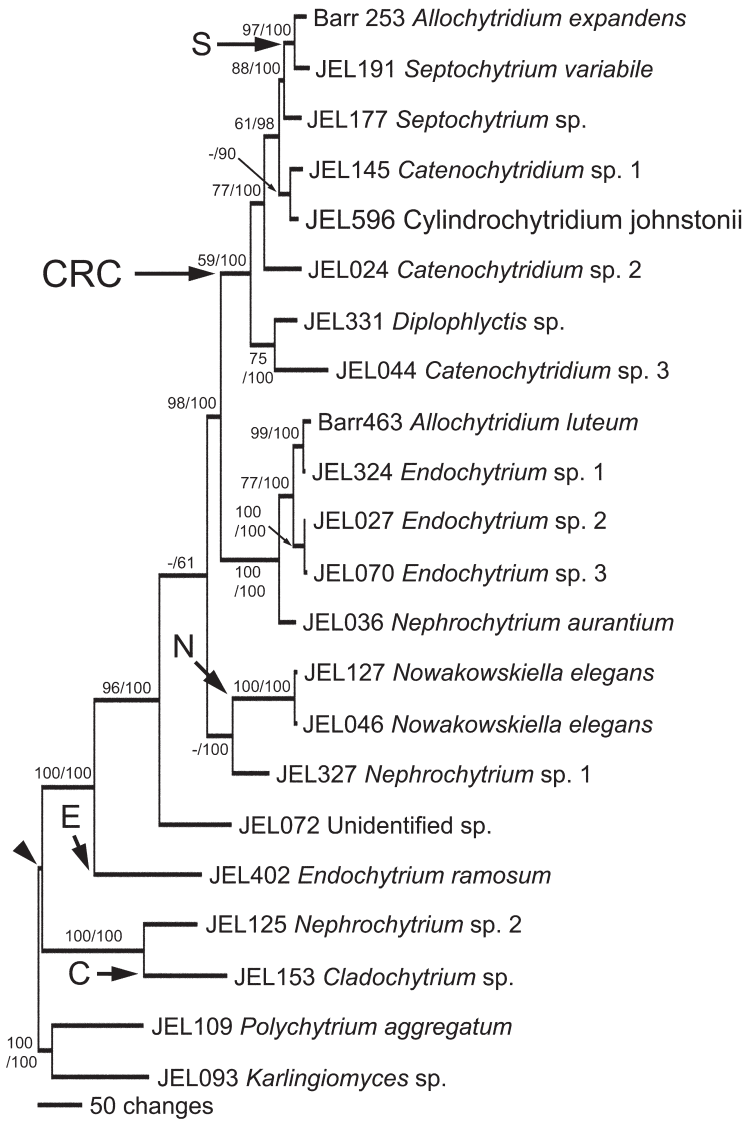

Figure 2. Majority rule consensus Bayesian phylogram of combined nucSSU and nucLSU rDNA for 20 taxa of *Cladochytriales* and 2 isolates in the *Polychytrium* clade (James et al. 2006). Labeled nodes correspond to the catenulated rhizoids clade (CRC) and families within the *Cladochytriales* (arrowhead): *Septochytriaceae* (S), *Nowakowskiellaceae* (N), *Endochytriaceae* (E), and *Cladochytriaceae* (C) (Mozley-Standridge et al. 2009). Branch support values are listed as maximum parsimony bootstrap values / Bayesian posterior probabilities. Tree length = 1234, CI = 0.6183, RI = 0.6235.

Karling (1941) illustrated elongation of *C. johnstonii* thalli as beginning during growth of the germling, with some zoosporangia having basal swellings. The developing zoosporangia we examined on agar were spherical (Fig. 1B) and grew an apical elongation only when nearly mature (Fig. 1E, F, I). Additionally, young thalli of our isolate on onionskin were also spherical (Fig. 1D).

Considering that Karling (1941) observed *C. johnstonii* in gross culture and we studied our isolate in pure culture, our observations agree well with his drawings, which constitute the type of *C. johnstonii*. *Cylindrochytridium endobioticum* Willoughby (Willoughby 1964) is the only other species in the genus, but Karling (1977) considered it to be a dubious member because of its considerable variation from *C. johnstonii*. Our genetic information provides the basis for adding additional species to the genus as well as evaluating the suitability of the genus for *C. endobioticum*, when it is found and brought into culture.

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